The EMBO Journal vol.12 no.1 pp.75–85, 1993

Human Wee1 kinase inhibits cell division by phosphorylating p34^{cdc2} exclusively on Tyr15

Clare H.McGowan and Paul Russell

Departments of Molecular and Cell Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

Communicated by P.Nurse

In fission yeast, the M-phase inducing kinase, a complex of p34^{cdc2} and cyclin B, is maintained in an inhibited state during interphase due to the phosphorylation of Cdc2 at Tyr15. This phosphorylation is believed to be carried out primarily by the Wee1 kinase. In human cells the negative regulation of p34^{cdc2}/cyclin B is more complex, in that Cdc2 is phosphorylated at two inhibitory sites, Thr14 and Tyr15. The identities of the kinases that phosphorylate these sites are unknown. Since fission yeast Wee1 kinase behaves as a dual-specificity kinase *in vitro*, a popular hypothesis is that a human Wee1 homolog might phosphorylate p34^{cdc2} at both sites. We report here that a human gene, identified as a possible Wee1 homologue, blocks cell division when overexpressed in HeLa cells. This demonstrates functional conservation of the Wee1 mitotic inhibitor. Contrary to the dual-specificity kinase hypothesis, purified human Wee1 phosphorylates p34^{cdc2} exclusively on Tyr15 *in vitro*; no Thr14 phosphorylation was detected. Human and fission yeast Wee1 also specifically phosphorylate synthetic peptides at sites equivalent to Tyr15. Mutation of a critical lysine codon (Lys114) believed to be essential for kinase activity abolished both the *in vivo* mitotic inhibitor function and *in vitro* kinase activities of human Wee1. These results conclusively prove that Wee1 kinases inhibit mitosis by directly phosphorylating p34^{cdc2} on Tyr15, and strongly indicate that human cells have independent kinase pathways directing the two inhibitor phosphorylations of p34^{cdc2}.

Key words: cell cycle/cyclin B/Cdc2/phosphorylation/Wee1

Introduction

Perhaps the most intensely studied aspect of cell cycle regulation has been the process governing the onset of mitosis. An important milestone in this analysis was the discovery that M-phase is brought about through the activation and subsequent actions of a heterodimeric protein kinase, consisting of a 34 kDa catalytic subunit encoded by *cdc2* and a ~60 kDa protein known as cyclin B (Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Draetta *et al.*, 1989; reviewed by Nurse, 1990). Genetic and biochemical studies of the fission yeast, *Schizosaccharomyces pombe*, have played a leading role in identifying the controls regulating p34^{cdc2}/cyclin B. Of particular importance was the discovery that activation of p34^{cdc2}/cyclin B is achieved by dephosphorylation of a critical tyrosyl residue (Tyr15) of p34^{cdc2} (Gould and Nurse,1989; Gould *et al.*, 1990). In fission yeast this reaction is predominantly catalyzed by Cdc25, a novel protein phosphatase originally identified as an essential rate-limiting inducer of mitosis (Russell and Nurse, 1986; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b; Strausfeld *et al.*, 1991; Lee *et al.*, 1992; Millar and Russell, 1992). Genetic data have shown that the Cdc25 mitotic inducer functions in opposition to the mitotic inhibitor encoded by *wee1* (Fantes, 1979; Russell and Nurse, 1986). The *wee1* gene was first identified by Nurse (1975), who found that inactivation of *wee1* caused cells to undergo mitosis at half the size of wild type, suggesting that Wee1 protein has an important role in a regulatory process delaying mitosis until cells have grown to the appropriate size. This idea was validated in later studies showing that introduction of extra copies of *wee1* into cells causes them to initiate mitosis at larger sizes that are directly related to *wee1* gene dosage (Russell and Nurse, 1987).

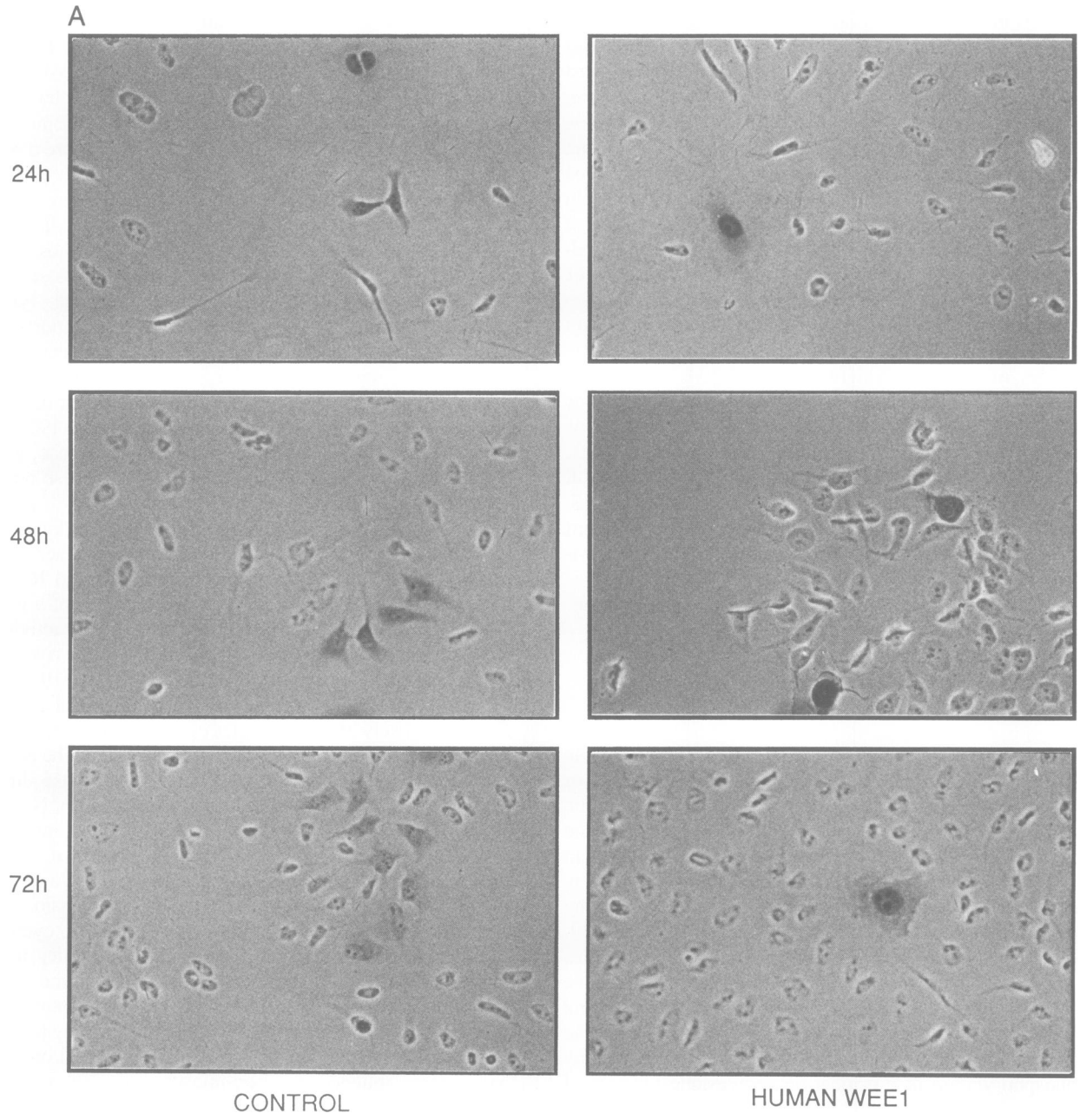
The observations that p34^{cdc2} is inhibited by tyrosyl phosphorylation and that *wee1* encodes a protein kinase that is counteracted at the genetic level by Cdc25 phosphatase have led to the suggestion that Wee1 kinase is closely and perhaps directly involved in promoting the tyrosyl phosphorylation of p34^{cdc2}. Indeed, two recent reports, one using fission yeast and the other an insect cell expression system, have described situations in which *in vivo* tyrosyl phosphorylation of p34^{cdc2} can be made dependent on Wee1 activity (Lundgren *et al.*, 1991; Parker *et al.*, 1991). In fission yeast this occurs when cells lack the *mik1* gene, which appears to be a redundant homolog of *wee1*. These reports followed the surprising discovery that p107^{wee1} kinase, although being most similar to Thr/Ser protein kinases in its sequence, actually has intrinsic Tyr and Thr/Ser kinase activity when assayed *in vitro* (Featherstone and Russell, 1991; Parker *et al.*, 1992). Wee1 is a member of a growing class of kinases that exhibit 'dual-specificity' activity *in vitro*, although the relevance of this activity to *in vivo* function is uncertain (reviewed by Lindberg *et al.*, 1992).

Analysis of p34^{cdc2}/cyclin B regulation in animal cells has followed closely after the studies of fission yeast. In certain key respects the regulation appears to be similar. The activity of p34^{cdc2}/cyclin B is inhibited during interphase due to phosphorylation of Tyr15 (Krek and Nigg, 1991a,b; Norbury *et al.*, 1991) and Cdc25 phosphatases play an essential role in inducing mitosis in human cells (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Millar *et al.*, 1991a; Nagata *et al.*, 1991). An important difference between fission yeast and animal cells is that Thr14 of p34^{cdc2} in cyclin B complexes is also phosphorylated and contributes to the inhibition of p34^{cdc2}/cyclin B kinase (Krek and Nigg, 1991a,b; Norbury *et al.*, 1991). The regulatory purpose of dual inhibitory phosphorylation is presently unknown. Initial studies using forms of p34^{cdc2} in which Thr14 and/or Tyr15 have been mutated to non-phosphorylatable residues,

© Oxford University Press

expressed in either *Xenopus* cell extracts or HeLa cells, have shown that only one site needs to be phosphorylated to prevent catastrophic premature mitosis (Krek and Nigg, 1991b; Norbury *et al.*, 1991). However, these studies were not designed to reveal less dramatic effects that could be critical for normal cell cycle control. In particular, dual kinase pathways inhibiting the activity of $p34^{cdc2}$/cyclin B could serve to respond differently to the array of signals impinging on the mitotic control, such as DNA replication and repair checkpoint controls (reviewed by Hartwell and Weinert, 1989; Enoch and Nurse, 1991), coordination of division with growth and response to growth factors.

A sensible way to address these issues is to identify the kinases that phosphorylate Thr14 and Tyr15 of $p34^{cdc2}$ in human cells. Because fission yeast Wee1 is a dual-specificity kinase *in vitro*, one important possibility is that both phosphorylations are carried out by a human Wee1 homolog. The cloning of a human gene that is potentially a homolog of fission yeast *wee1* has recently been reported (Igarashi *et al.*, 1991). Strong overexpression of this gene rescues lethal premature mitosis mutants in fission yeast. Although genes other than *wee1* homologs have also been found to do this (Bueno and Russell, 1992), the fact that the human gene encodes a potential protein kinase having a catalytic domain that is somewhat more similar to Wee1 and Mik1 than to most other kinases suggested that the human '*wee1*-like' gene might be an authentic *wee1* homolog. Two key predictions should be fulfilled if this is true. The first is that the human gene should delay or block mitosis when overexpressed in human cells. The second prediction is that both the human and Wee1 kinases should act as cell division inhibitors by a common mechanism, probably by phosphorylating $p34^{cdc2}$ on Tyr15. In this communication we test these proposals and additionally determine whether human Wee1 phosphorylates $p34^{cdc2}$ on Thr14 as well as Tyr15.



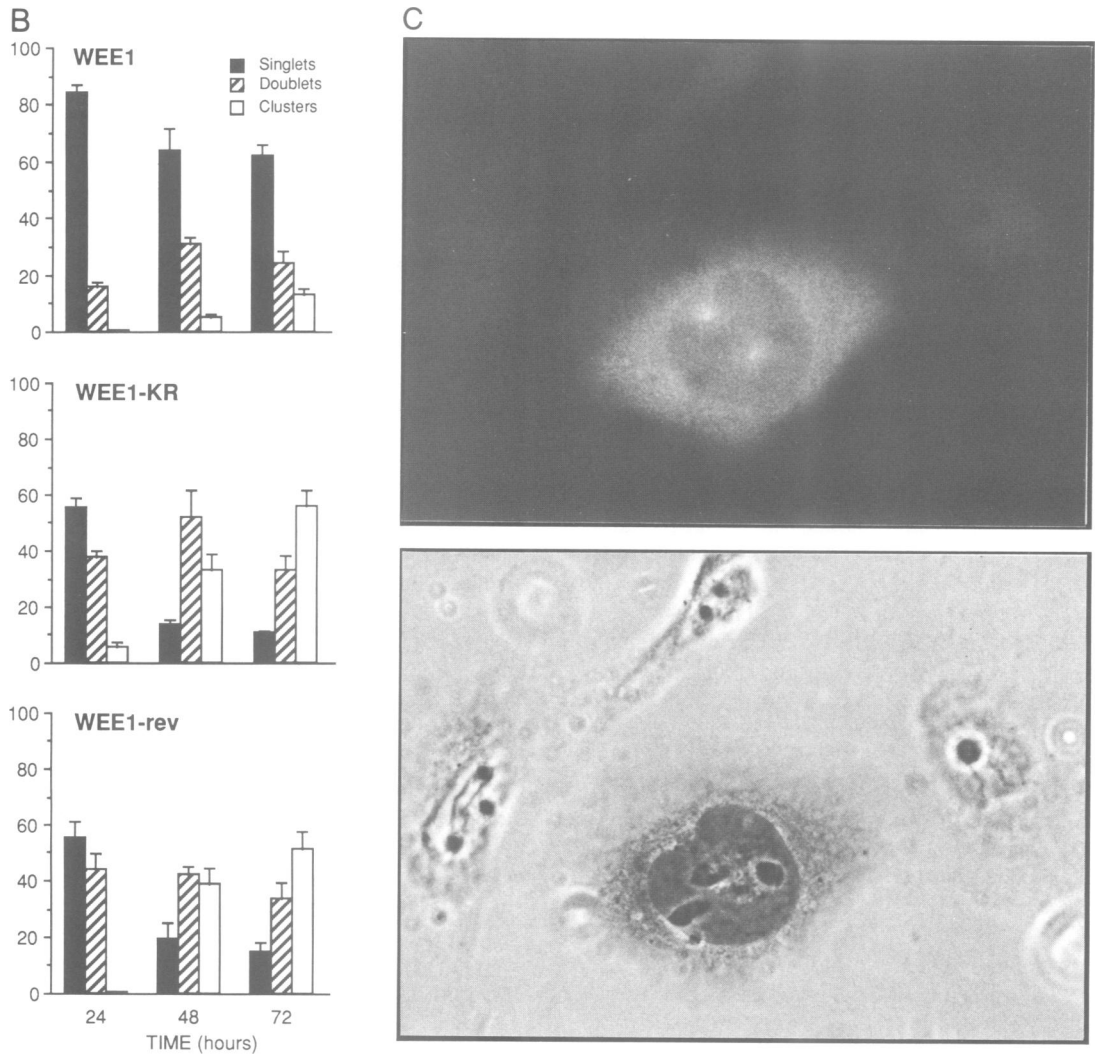


Fig. 1. Overexpression of human *WEE1* inhibits cell division in HeLa cells. The human *WEE1* cDNA was cloned behind the α-globin promoter in an expression plasmid suitable for transfection of HeLa cells. Co-transfections were performed with the same vector containing the β-galactosidase gene. Three forms of human *WEE1* were used, *WEE1* in the correct orientation with respect to the α-globin promoter, *WEE1-KR* in which a critical lysine was replaced with arginine, thereby inactivating the kinase, and *WEE1-rev* in which the *WEE1* cDNA was present in the reverse orientation with respect to the α-globin promoter. (**A**) Phase contrast photographs of representative fields of X-gal stained transfected HeLa cells fixed 24, 48 and 72 h after plating. Left panels show transfectants having *WEE1-rev*, right panels show transfectants expressing *WEE1*. The majority of control transfectants had undergone more than two rounds of division by 72 h, whereas most of the transfectants expressing *WEE1* had failed to undergo a single division by this time. Note that control transfectants in the later time points stained less intensely as a result of plasmid dilution during divisions. (**B**) Quantification of X-Gal positive cells as either single cells (■), doublets (▨) and clusters of three or more cells (□). At least 200 single cells, doublets or clusters were counted for each data point. The data represent the mean ± SEM of six independent transfections for the *WEE1* construct, and three independent transfectants for each of the control constructs. (**C**) Anti-cyclin B staining of *WEE1* transfected cells from 72 h time point. Top panels shows pattern of cyclin B staining, lower panel shows phase contrast image of the same field.

Results

Human Wee1 kinase inhibits cell division in human cells

At the outset of this study it was important to confront the issue of whether the putative human *wee1*-like gene described by Igarashi *et al.* (1991) functioned in human cells in a homologous fashion to *wee1* in fission yeast. A critical part of the proof that fission yeast *wee1* encoded a mitotic inhibitor was the demonstration that high overexpression of *wee1* caused a cell cycle arrest phenotype (Russell and Nurse, 1987). An important feature of those experiments was the observation that while *wee1* overexpression inhibited cell division, it did so without greatly affecting the rate of increase of cell mass, such that cells grew extremely large during the arrest. This demonstrated that Wee1 specifically blocked cell cycle progression, as opposed to inhibiting cell division by a non-specific process involving cellular toxicity.

If the human gene described by Igarashi *et al.* (1991) is a true *wee1* homolog, then overexpression of this gene in human cells should cause a cell cycle arrest analogous to that observed in *wee1* overexpression studies carried out in fission and budding yeasts (Russell and Nurse, 1987; Russell *et al.*, 1989). We decided to test this idea by the transient co-transfection method using HeLa cells. Human *WEE1* cDNA was cloned in both orientations into an expression vector (pCMUIV) suitable for transient transfection of HeLa cells (see Materials and methods). This vector uses a globin gene promoter. In half of our experiments our control plasmid was pWEE1-rev, having *WEE1* in the reverse orientation to the globin promoter. In a second series of experiments we used as a control the plasmid pWEE1-KR,

in which a mutant version of *WEE1* was placed in the correct orientation in the pCMUIV vector. This mutation, changing codon Lys114 to Arg114, was created by site-directed mutagenesis. Lys114 corresponds to a residue that is absolutely conserved in all known protein kinases and has been found to be essential for kinase activity in all cases examined (Hunter and Cooper, 1985). Mutation of the corresponding lysine codon in fission yeast *wee1* abolished both the *in vitro* kinase activity of p107^{wee1} and its ability to delay the onset of mitosis in yeast (Russell *et al.*, 1989; Featherstone and Russell, 1991). To provide a simple method for identifying transfected cells, a pCMUIV vector expressing the *Escherichia coli* β-galactosidase gene was co-transfected in each experiment. After 24 h in the presence of $CaPO_4$−DNA precipitates, co-transfected cells were washed, removed from plates by trypsinization and seeded at a low density on cover slips. At 24 h intervals after seeding, cells were fixed and stained for β-galactosidase activity using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). At this density of seeding, independently transfected cells were rarely near to each other, such that almost all adjacent stained cells arose from a single transfectant. Singlets, doublets and clusters of three or more stained cells were scored. At least 200 events were scored at every time point in each of six experiments. In three of the experiments the *WEE1-rev* control was used, while in the other three experiments the control was *WEE1-KR*. In all six experiments the frequency of X-Gal positive cells or cell clusters was similar at all time points in parallel transfections, indicating that *WEE1* expression reduced neither the efficiency of transfection nor the production of β-galactosidase.

The six experiments gave highly consistent results, showing in each case that *WEE1* expression specifically inhibited cell division. Representative fields for each time point in one experiment are shown in Figure 1A. In the *Wee1-rev* transfected cells, cell doublets were seen by 24 h, clusters of four cells were seen at 48 h, and by 72 h clusters of approximately eight cells were commonly seen. Note that by 72 h the X-Gal staining of control transfectants was becoming low due to plasmid dilution during division. By contrast, at all three time points the majority of *WEE1* transfectants appeared as single large cells that stained quite strongly with X-Gal (Figure 1A). The tabulated data for all six experiments are presented in Figure 1B. At each of the three time points it is clear that *WEE1* expression significantly inhibited cell division relative to both controls. For example, by 72 h 52% of the *WEE1-rev* transfectants and 58% of the *WEE1-KR* transfectants had formed clusters of cells, while at the same time point $<13\%$ of the *WEE1* transfectants had formed clusters of three or more cells and $>60\%$ had failed to undergo a single division (Figure 1B).

A number of observations indicate that the phenotype caused by *WEE1* overexpression in HeLa is analogous to that caused by overexpression of fission yeast *WEE1* in yeast, namely continued cellular growth in the absence of cell division. Of particular importance is the fact that most of the arrested *WEE1* transfected cells were quite large. Size estimations made using an eyepiece micrometer indicated that ~90% (30/33) of the single cells expressing *WEE1* were greater than twice the size of untransfected cells at 72 h, whereas only ~2% (2/107) of the *Wee1-rev* control transfectants at 72 h were of this size. Secondly, the observation that large *WEE1* transfectants remained attached to the cover slip with normal morphology further indicated that the cell division arrest was not due to any non-specific toxic effect. Thirdly, the fact that *WEE1* transfectants stained intensely with X-Gal showed that β-galactosidase expression continued during the division arrest. Finally, because the *WEE1-KR* mutation abolished the cell cycle arrest phenotype (Figure 1B), we can conclude that these phenotypes were due to Wee1 kinase activity.

The arrested *WEE1* transfectants remained as flat cells, as opposed to the rounded phenotype of cells in mitosis, indicating that cell cycle progression was blocked at a point prior to the onset of M-phase. This further supported by the fact that all of the arrested *WEE1* transfectants had uncondensed chromosomes typical of interphase cells. As mentioned above, if human *WEE1* is an authentic homolog of fission yeast *wee1*, then the *WEE1* transfectants would be expected to be arrested in late G_2 phase. To determine whether this was so, we took advantage of the observations of Pines and Hunter (1989, 1991) who showed that cells in late G_2 have high levels of cyclin B in the cytoplasm, concentrated in the perinuclear region. Cyclin B is undetectable prior to S-phase. Staining with affinity purified antibody to human cyclin B showed that ~90% (27/30) of the arrested *WEE1* transfectants at the 72 h time point had accumulated very high levels of cyclin B in the cytoplasm. The anti-cyclin B staining pattern of a typical *WEE1* transfectant is shown in Figure 1C. By contrast, only ~5% (5/105) of the *WEE1-rev* control transfectants stained brightly with anti-cyclin B antibody. Although we cannot eliminate the possibility that *WEE1* overexpression uncoupled cyclin B production from normal cell cycle controls, the most reasonable interpretation of these observations is that *WEE1* functions as a mitotic inhibitor in human cells. This is fully consistent with the demonstration that human *WEE1* rescues mitotic catastrophe mutants in fission yeast (Igarashi *et al.*, 1991).

Human Wee1 protein has dual-specificity kinase activity in vitro

Our next goal was to determine the biochemical mechanism by which human Wee1 protein functions as a cell division inhibitor. We reasoned that it would be essential to have a purified and active form of Wee1 in order to define its biochemical function precisely. Initial attempts to express active human Wee1 in bacteria were unsuccessful, therefore we constructed a plasmid designed to express Wee1 fused to the C-terminus of gluathione-S-transferase when introduced into Schneider cells, a *Drosophila* cell line. The fusion protein GST−Wee1, eluted from a GSH−Sepharose affinity column, migrated with the predicted molecular weight of 72 kDa in SDS−PAGE (Figure 2A). Purified GST−Wee1 became phosphorylated in standard protein kinase assay conditions (Figure 2B). Two-dimensional phosphoamino acid (2D-PAA) analysis of auto-phosphorylated GST−Wee1 revealed that it contained mainly phosphotyrosine with a small amount (~5%) of phosphoserine (Figure 2C). This result is similar to one previously made with *S.pombe* p107^{wee1} that was found to autophosphorylate equally on tyrosyl and seryl residues *in vitro* (Featherstone and Russell, 1991). It is likely that autophosphorylation occurs on the Wee1 polypeptide, since

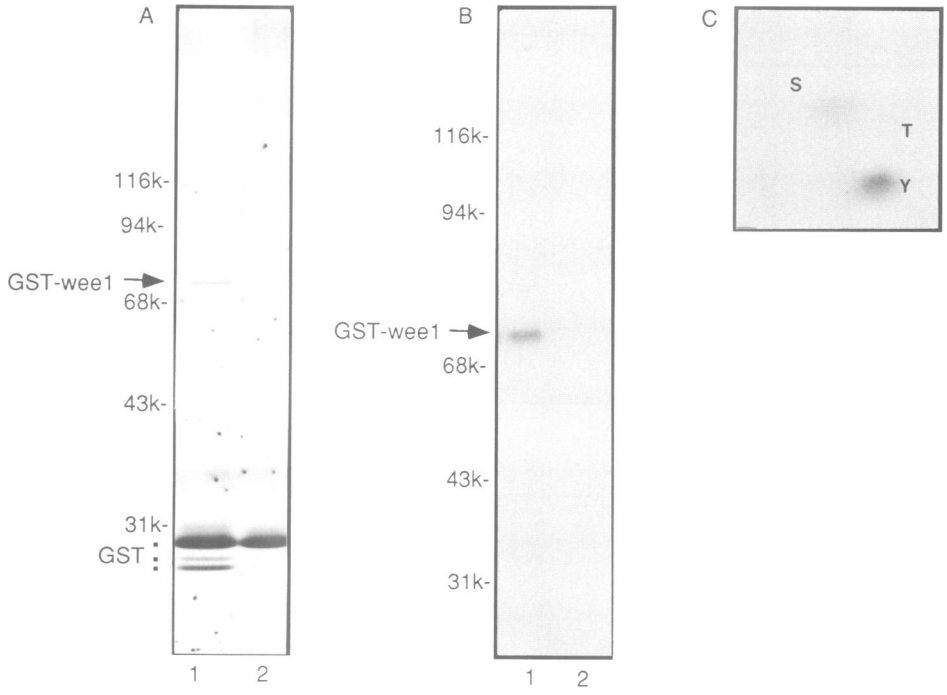


Fig. 2. Purified GST−Wee1 autophosphorylates on tyrosine and serine. (**A**) GST−Wee1 (lane 1) and GST (lane 2) produced in Schneider cells were purified by GSH−Sepharose affinity chromatography, eluted with excess GSH, subjected to SDS−PAGE and silver stained. GST−Wee1 migrated as a 72 kDa protein, which is the predicted molecular weight of the fusion protein. Vector derived GST was detected as a doublet of 24 kDa. Endogenous GST was detected in both samples. (**B**) GST−Wee1 (lane 1) and GST (lane 2) were incubated with [γ-^{32}P]ATP for 15 min at 30°C, the reaction was terminated by addition of SDS sample buffer, boiled and subjected to SDS−PAGE. The dried gel was autoradiographed. GST−Wee1 was detected as a phosphoprotein of 72 kDa. (**C**) Phospho-amino acid analysis of autophosphorylated GST−Wee1 revealed that it contains predominantly phosphotyrosine together with a low level of phosphoserine. Positions of phospho-amino acid standards are shown: S, serine; T, threonine; T, tyrosine.

we have never observed transphosphorylation of free GST in our assays.

We further investigated the kinase activity of GST−Wee1 using a variety of substrates. GST−Wee1 failed to phosphorylate mixed histones, myelin basic protein, enolase and the synthetic mixture poly(Glu/Tyr). However, the short peptide angiotensin II (sequence DRVYVHPF), which had previously been shown to be phosphorylated by fission yeast p107^{wee1} (Featherstone and Russell, 1991), was also a substrate of GST−Wee1 (Figure 3A). 2D-PAA analysis confirmed that angiotensin was exclusively phosphorylated on tyrosine by GST-Wee1 (Figure 3B). These data indicate that tyrosine kinase activity is a conserved feature of Wee1 proteins.

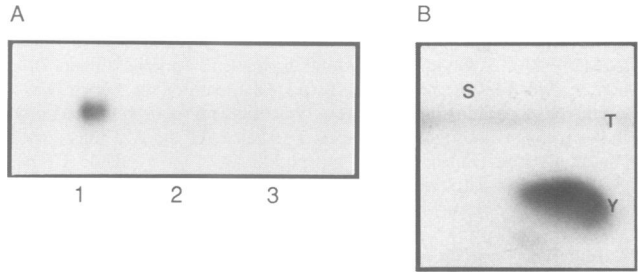


Fig. 3. GST−Wee1 phosphorylates angiotensin II on tyrosine. (**A**) Kinase assays were performed using GST−Wee1 plus angiotensin (lane 1), GST plus angiotensin (lane 2) or GST−Wee1 alone (lane 3). Products were resolved by thin-layer electrophoresis at pH 1.9. (**B**) Phospho-amino acid analysis of angiotensin phosphorylated by GST−Wee1 revealed only phosphotyrosine.

Human Wee1 kinase phosphorylates the Cdc2 subunit of p34^{cdc2}/cyclin B specifically on Tyr15

The regulation of p34^{cdc2}/cyclin B kinase in higher eukaryotes involves the phosphorylation of residues Thr14 and Tyr15 on the p34^{cdc2} molecule (Krek and Nigg, 1991a,b; Norbury *et al.*, 1991). Human Wee1 could act as a mitotic inhibitor by phosphorylating p34^{cdc2} on one or both of these critical sites, therefore we examined whether these sites were phosphorylated by GST-Wee1 *in vitro*. Human p34^{cdc2} and cyclin B were co-expressed and purified as a complex from baculovirus infected Sf9 cells (see Materials and methods). The Cdc2 subunit of this form of p34^{cdc2}/cyclin B proved to be a substrate for GST−Wee1 kinase (Figure 4A). Phospho-amino acid analysis of p34^{cdc2} from this gel showed mainly phosphotyrosine with a low level (~10%) of phosphothreonine (Figure 4B). In five separate experiments the level of phosphothreonine was variable but always <20% of total c.p.m., and in one case no phosphothreonine was detected. Phosphoserine was never observed.

Peptide mapping following trypsin treatment of the phosphorylated p34^{cdc2} generated one major phosphopeptide (labeled A in Figure 4C, panel 1). The migration characteristics of this peptide were very similar to those of the peptide having Tyr15 phosphorylated, generated from p34^{cdc2} labeled *in vivo* (Gould and Nurse, 1989). Indeed, peptide A was phosphorylated exclusively on tyrosine (Figure 4C, panel 1A). To confirm the identity of peptide A, the 2D tryptic map of p34^{cdc2} phosphorylated by GST−Wee1 was compared with that of a synthetic peptide

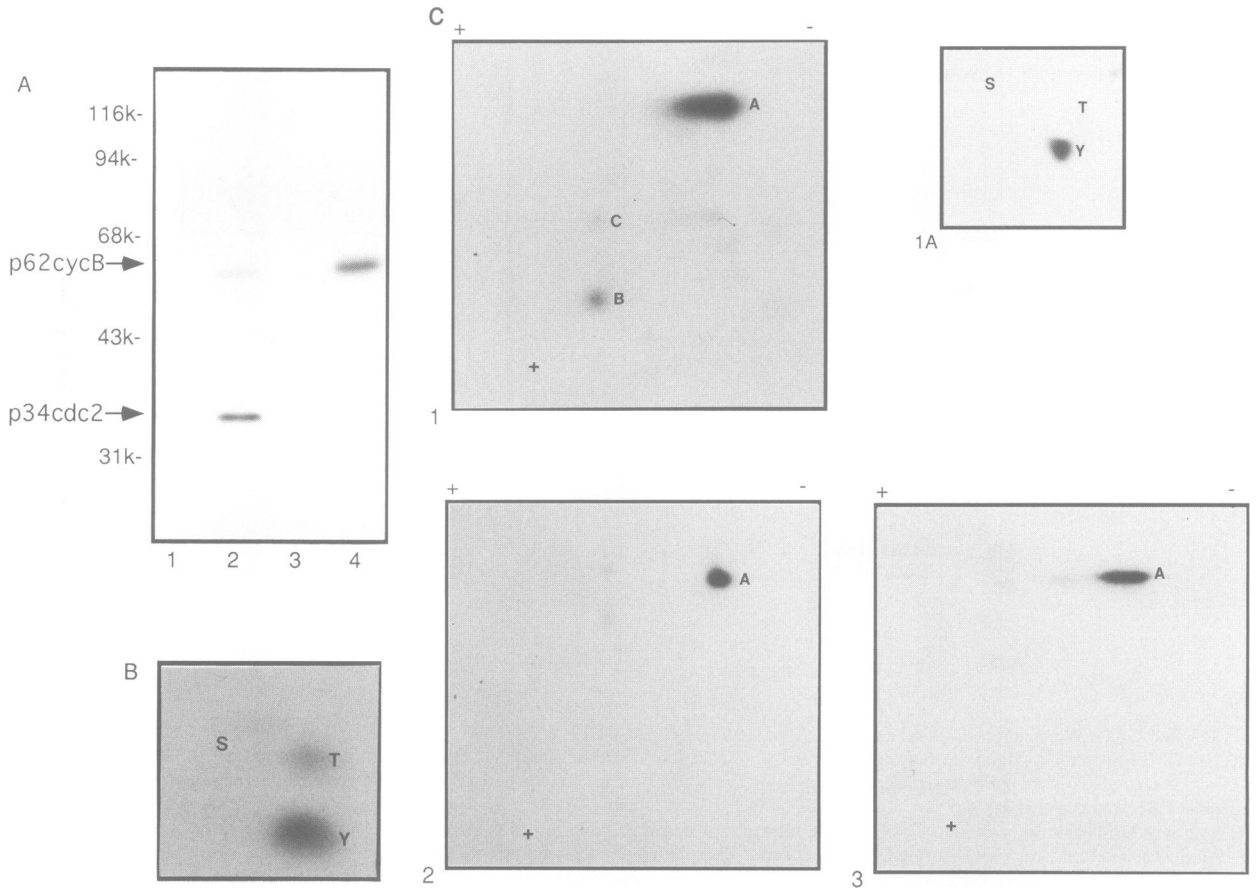


Fig. 4. GST−Wee1 phosphorylates the cdc2 subunit of $p34^{cdc2}$/cyclin B purified from Sf9 cells. (**A**) Kinase assays were performed using GST−Wee1 alone (lane 1), GST−Wee1 plus $p34^{cdc2}$/cyclin B (lane 2), GST alone (lane 3), GST plus $p34^{cdc2}$/cyclin B (lane 4). Reactions were terminated, proteins denatured and $p34^{cdc2}$ was precipitated using an antibody directed against the C-terminus of human cdc2. Samples were resolved by SDS−PAGE. Under these conditions a small, variable amount of cyclin-B ($p62^{cycB}$) reassociates with $p34^{cdc2}$ (lane 4). (**B**) Phosphorylated $p34^{cdc2}$ was excised from the gel shown in (A). One-tenth of the sample was subjected to 2D-PAA analysis, revealing predominantly phosphotyrosine and a low level of phosphothreonine. (**C**) The remaining phosphorylated $p34^{cdc2}$ was digested with trypsin and analyzed by 2D peptide mapping. Panel 1 shows a major phosphopeptide species (labeled A) and two minor spots (labelled B and C); Panel 1A is a 2D-PAA analysis of peptide A, revealing phosphotyrosine. Panel 2 shows a peptide map of the synthetic TY peptide phosphorylated on Tyr15 with $p60^{src}$ and digested with trypsin. The $p60^{src}$-phosphorylated peptide (IGEGTYGVVYK) co-migrated with peptide A, as confirmed in panel 3 showing a peptide map of peptide A mixed with an equal amount of radiolabeled $p60^{src}$-phosphorylated tryptic peptide. Phosphopeptides derived from chymotrypsin treatment of the $p60^{src}$-phosphorylated peptide in panel 2 and peptide A from panel 1 also co-migrated (data not shown), proving that peptide A is IGEGTYGVVYK phosphorylated on Tyr15. The orientation of the electrodes is shown; electrophoresis was at pH 1.9. Ascending chromatography was in phosphochromatography buffer (Boyle *et al.*, 1991).

(TY peptide) corresponding to residues 7−26 of $p34^{cdc2}$. The TY peptide was phosphorylated by $p60^{src}$ tyrosine protein kinase, which phosphorylates this peptide only at Tyr15 (Gould and Nurse, 1989; Krek and Nigg, 1991a; Norbury *et al.*, 1991). The 2D map showed that $p60^{src}$ tryptic phosphopeptide (IGEGTYGVVYK) migrated in an identical fashion to peptide A (Figure 4C, panel 2), as confirmed by mixing the two phosphopeptides (Figure 4C, panel 3). The two phosphopeptides also co-migrated following treatment with chymotrypsin (data not shown), proving that peptide A was phosphorylated on Tyr15. The failure to detect any phosphothreonine in peptide A indicates that GST−wee1 did not generate $p34^{cdc2}$ singly phosphorylated on Thr14.

Two minor spots that constituted <5% of total c.p.m. were also detected in the tryptic map of this sample of $p34^{cdc2}$ phosphorylated by GST−Wee1 (Figure 4C, panel 1). There was insufficient label in either spot to detect phospho-amino acids. Thus we could not determine whether either of these spots might correspond to the tryptic peptide having both Thr14 and Tyr15 phosphorylated.

To establish more firmly whether human Wee1 directly phosphorylates $p34^{cdc2}$, and to investigate further which sites it phosphorylates, we used a second source of $p34^{cdc2}$/cyclin B as a substrate. Human $p34^{cdc2}$/cyclin B was immunoprecipitated from M-phase arrested Hela cells using an anti-cyclin B antibody. The $p34^{cdc2}$/cyclin B immunocomplex was treated with the irreversible kinase inactivator *p*-fluorosulfonylbenzoyl adenosine (FSBA) in order to reduce kinase activities present in the immunocomplex (Zoller *et al.*, 1981). As assayed by cyclin B phosphorylation, FSBA treatment reduced immunocomplex kinase activity by ~98%. Incubation of the FSBA-treated immunocomplex with GST−Wee1 under kinase assay conditions resulted in phosphorylation of a 34 kDa protein (Figure 5A, lane 1). In separate experiments the phosphorylation products were denatured and immunoprecipitated with an anti-Cdc2 antibody to confirm that the

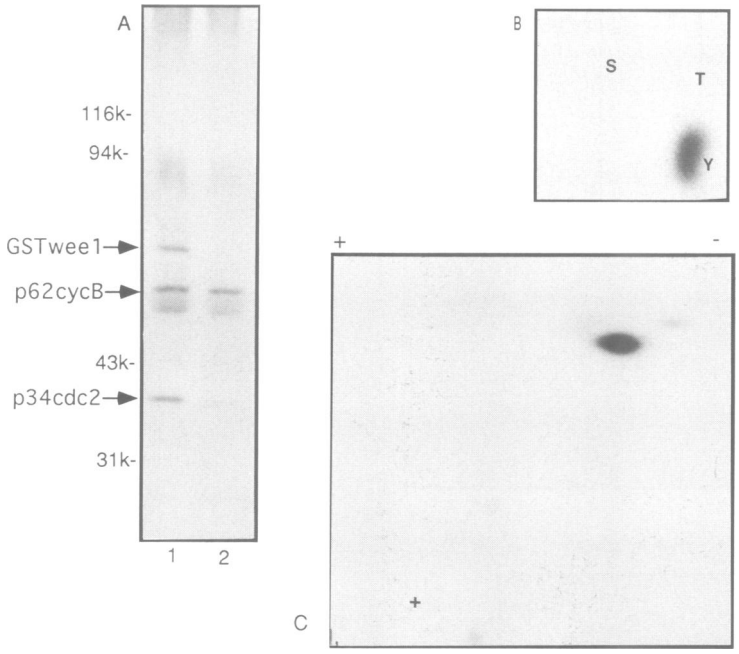


Fig. 5. GST−Wee1 phosphorylates the cdc2 subunit of $p34^{cdc2}$/cyclin B isolated from HeLa cells. (**A**) Anti-cyclin B immunocomplexes from nocodazole arrested HeLa cells were incubated with 1 mM FSBA for 90 min at 30°C in order to inhibit $p34^{cdc2}$/cyclin B kinase. This $p34^{cdc2}$/cyclin B was then added to kinase assays having GST−Wee1 (lane 1) or GST (lane 2). Total products were separated by SDS−PAGE and autoradiographed. Phosphorylated $p34^{cdc2}$ and GST−Wee1 are seen in lane 1. Note that FSBA failed to inactivate completely the $p34^{cdc2}$/cyclin B kinase and thus a low level of phosphorylated $p62^{cycB}$ was detected in both lanes. (**B**) Phospho-amino acid analysis of $p34^{cdc2}$ revealed that it was phosphorylated on Tyr. (**C**) Phosphorylated $p34^{cdc2}$ was digested with trypsin and analyzed by 2D peptide mapping. Peptide A contained >98% of the total c.p.m. Conditions were identical to those described above. The identity of peptide A was confirmed by co-migration with synthetic peptide labeled at Tyr15 (not shown).

34 kDa protein was $p34^{cdc2}$ (data not shown). Phosphorylated GST−Wee1 and cyclin B were also detected (Figure 5A, lane 1). Phosphorylation of cyclin B was probably due to residual $p34^{cdc2}$/cyclin B kinase activity (see control assay lacking GST−Wee1 in lane 2). No other phosphorylated proteins were detected, strongly arguing that GST−Wee1 was directly responsible for $p34^{cdc2}$ phosphorylation. This was further supported by the fact that treatment of the immunocomplex with FSBA had no impact on the level of $p34^{cdc2}$ phosphorylation promoted by GST−Wee1 (data not shown). Phospho-amino acid analysis revealed that $p34^{cdc2}$ was phosphorylated exclusively on tyrosine (Figure 5B). Phosphopeptide mapping following trypsin treatment of the phosphorylated $p34^{cdc2}$ revealed a single major spot (Figure 5C) which migrated exactly with the characteristics of the trypsin-treated TY peptide phosphorylated on Tyr15 and which contained only phosphotyrosine (data not shown). These data provide stronger evidence that human Wee1 specifically phosphorylates Tyr15 of $p34^{cdc2}$.

Human Wee1 kinase phosphorylates synthetic peptides at sites corresponding to pTyr15 of $p34^{cdc2}$

To establish more conclusively whether human Wee1 has the intrinsic ability to phosphorylate Tyr15 of $p34^{cdc2}$ without the enzymatic participation of other proteins, we next tested the ability of GST−Wee1 to phosphorylate a series of synthetic peptides corresponding to the region encompassing Tyr15 of $p34^{cdc2}$. We found that the TY peptide described above was a substrate for GST−Wee1 kinase (Figure 6A). 2D-PAA revealed only phosphotyrosine (Figure 6B). This peptide was readily dephosphorylated by a tyrosine specific phosphatase (Figure 6D, lane 3) but not by a Ser/Thr phosphatase (Figure 6D, lane 1). A slightly shorter peptide corresponding to residues 6−20 of human cdc2 also acted as a substrate when assayed using GST−Wee1 (Figure 6C, lane 2). However, a similar peptide in which Tyr15 was substituted with Phe was unable to act as a substrate (Figure 6C, lane 3). Two other mutant peptides in which Thr14 was substituted with Ser (Figure 6D, lane 4) and Tyr19 was substituted with Lys (Figure 6D, lane 5) also acted as substrates for GST−Wee1. This proves that Tyr19 is not required for phosphorylation of these peptides by Wee1. The observation that relevant synthetic peptides act as substrates for GST−Wee1 strengthens the hypothesis that Wee1 directly phosphorylates Tyr15 of $p34^{cdc2}$, that no other protein is required for this reaction, and that Thr14 is not a target of GST−Wee1.

Mutation of a critical lysine codon abolishes Wee1 kinase activity

The peptide phosphorylation experiments proved that GST−Wee1 preparations have the intrinsic ability to phosphorylate Tyr15 of Cdc2. We then wished to establish formally that this phosphorylation was specifically carried out by Wee1, as opposed to any contaminating kinase that otherwise had escaped detection. To do this we purified a mutant form of GST−Wee1 that had the Arg114 codon substituted for Lys114. As described above, on the basis of studies with many other kinases, including fission yeast $p107^{wee1}$, this mutation could be expected to inactivate Wee1 kinase. Equal amounts of GST−Wee1 and mutant GST−Wee1-KR were produced and purified in parallel and then tested for their ability to phosphorylate TY peptide. As

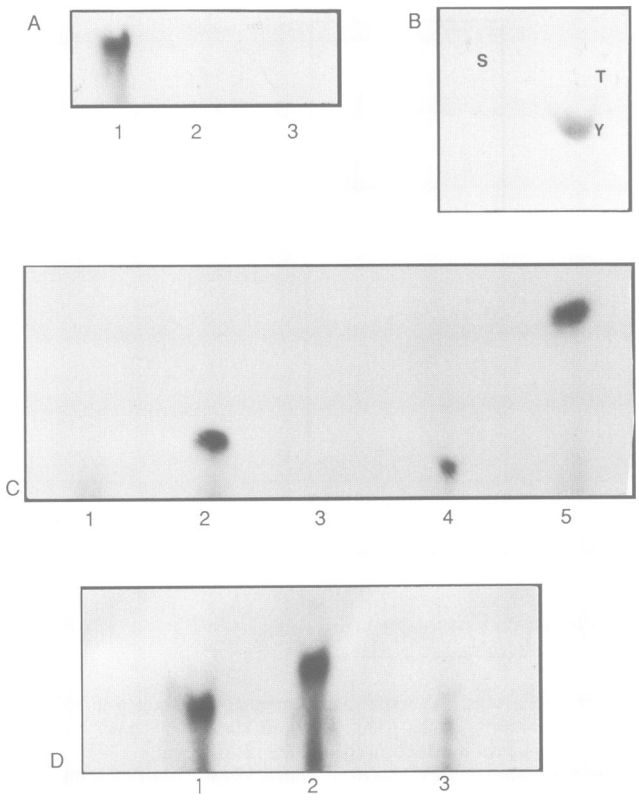


Fig. 6. GST−Wee1 phosphorylates cdc2-TY peptides. (**A**) The products of kinase assays containing GST−Wee1 plus TY peptide (lane 1), GST plus TY peptide (lane 2) or GST−Wee1 alone (lane 3) resolved by thin-layer electrophoresis revealed that GST−Wee1 phosphorylated the TY peptide. (**B**) 2D-PAA of peptide recovered from TLC contained only phosphotyrosine. (**C**) The products of kinase assays containing GST−Wee1 alone (lane 1), plus wild type 6-20 peptide (lane 2), mutant F15 peptide (lane 3), mutant S14 peptide (lane 4) and mutant K19 peptide (lane 5) resolved by thin-layer electrophoresis revealed that mutation of Y15 prevents phosphorylation of this peptide by GST−Wee1. (**D**) Incubation of the peptide in the presence of purified protein phosphatase $2A_c$ (lane 1) or in buffer (lane 2) did not result in any significant level of dephosphorylation; T cell phosphatase (lane 3) resulted in significant dephosphorylation.

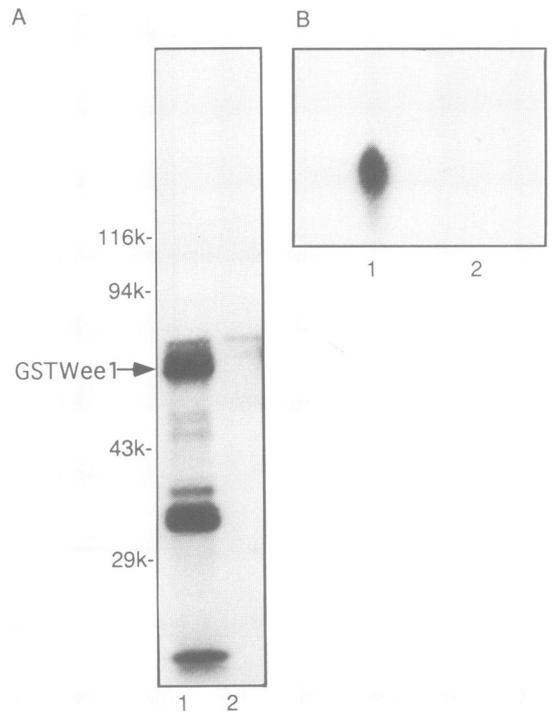


Fig. 7. Mutation of Lys114 abolishes Wee1 kinase activity. (**A**) Equal amounts of GST−Wee1 (lane 1) or GST−Wee1-KR, in which the Arg114 codon was replaced by Lys114 (lane 2), were tested for their ability to autophosphorylate exactly as described in Figure 2. No phosphorylation was detected in the GST−Wee1-KR sample. Several breakdown products were detected in this preparation of GST−Wee1. (**B**) The products of kinase assays containing GST−Wee1 plus TY peptide (lane 1) and GST−Wee1-KR plus TY peptide (lane 2) resolved by thin-layer electrophoresis revealed that GST−Wee1-KR was unable to phosphorylate this peptide.

shown in Figure 7, GST−Wee1-KR was completely unable to phosphorylate this peptide. This was due to inactivation of general kinase activity, since GST−Wee1-KR also was inactive in autophosphorylation assays (Figure 7). This provides conclusive proof that the ability of GST−Wee1 preparation to phosphorylate Tyr15 of Cdc2 was due to the direct actions of the Wee1 kinase.

S.pombe p107^{wee1} phosphorylates p34^{cdc2} and synthetic peptides in sites corresponding to Tyr15 of p34^{cdc2}

Previously we reported that *S.pombe* p107^{wee1} immunoprecipitated from Sf9 cells phosphorylated the peptide substrate angiotensin on tyrosine but we were unable to demonstrate phosphorylation of p34^{cdc2} by p107^{wee1} (Featherstone and Russell, 1991). We have recently developed a soluble source of p34^{cdc2} from *S.pombe*. Briefly, this was accomplished by producing, in fission yeast, a form of p34^{cdc2} that has six histidines at the C-terminus, followed by purification on nickel−agarose (M. Charbonneau, C.H.McGowan and P.Russell, manuscript in preparation). This preparation is active as a histone H1 kinase and thus must contain p34^{cdc2}/cyclin complex. We found that this form of p34^{cdc2} was a substrate for immunoprecipitated p107^{wee1} (Figure 8A). Although we did not map the site of phosphorylation, we did use the synthetic peptides described above to show that p107^{wee1} specifically phosphorylates sites corresponding to Tyr15 of p34^{cdc2} (Figure 8B). Substitution of tyrosine residue corresponding to Tyr15 abolished phosphorylation of the peptide, indicating that fission yeast p107^{wee1} also does not phosphorylate Thr14. This is consistent with failure to detect phosphorylated Thr14 in fission yeast p34^{cdc2} metabolically labeled *in vivo* (Gould and Nurse, 1989).

Discussion

Three important conclusions are derived from these studies. The first is that a human gene, first identified by its ability to rescue a mitotic catastrophe mutant strain of fission yeast (Igarashi *et al.*, 1991), is a functional homolog of fission yeast mitotic inhibitor encoded by *wee1*. High expression of this human *WEE1* gene in HeLa cells effectively blocked cell cycle progression while allowing cells to increase in size, causing essentially the same cell division cycle arrest phenotype as does overexpression of *wee1* or *mik1* in fission yeast (Russell and Nurse, 1987; Lundgren *et al.*, 1991). Importantly, the ability of human Wee1 protein to block cell cycle progression was abolished by alteration of a single conserved lysine residue that is essential for kinase activity.

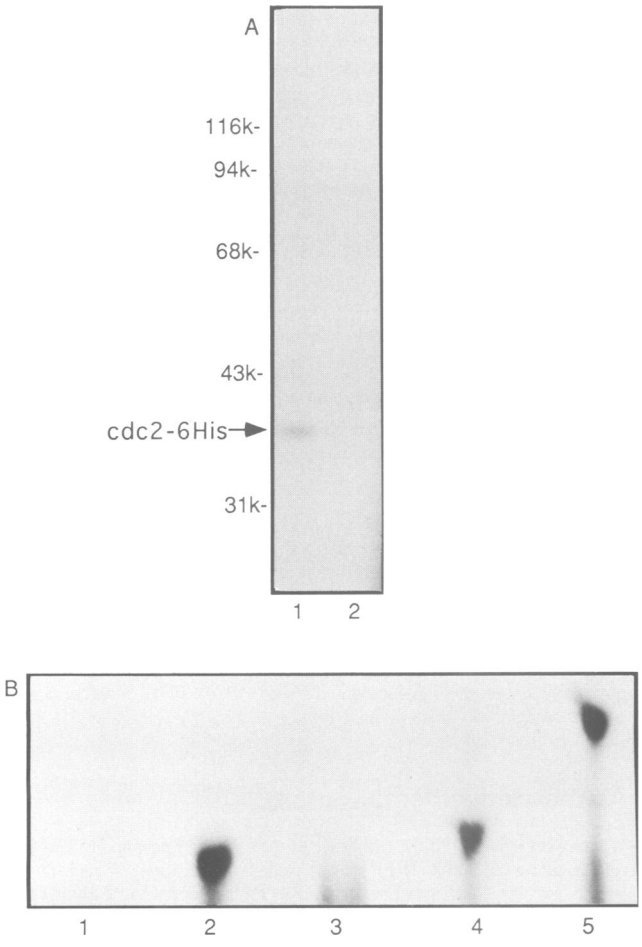


Fig. 8. *S.pombe* p107^{wee1} phosphorylates p34^{cdc2}/cyclin B derived from fission yeast and TY peptides. (**A**) A vector construct expressing *S.pombe* cdc2 with six histidine residues at the C-terminus (H.Charbonneua, C.H.McGowan and P.Russell, manuscript in preparation) was used to express p34^{cdc2}−6His in fission yeast. This protein was purified under native conditions using NTA−agarose (Qiagen), treated with FSBA as described above and then added to kinase assays containing p107^{wee1} immunoprecipitated from Sf9 cells infected with baculovirus producing *S.pombe* Wee1 (lane 1) or reaction buffer (lane 2). Reactions were terminated, proteins denatured and p34^{cdc2} was precipitated using an antibody directed against bacterially produced *S.pombe* p34^{cdc2}. Fission yeast p107^{wee1} was prepared as described by Featherstone and Russell (1991). (**B**) The products of kinase assays containing immunoprecipitated p107^{wee1} alone (lane 1), plus wild type 6-20 peptide (lane 2), mutant F15 peptide (lane 3), mutant S14 peptide (lane 4) and mutant K19 peptide (lane 5) resolved by thin-layer electrophoresis reveals that mutation of Y15 prevents phosphorylation of this peptide by *S.pombe* p107^{wee1}.

These data, coupled with the previous discoveries of human genes that are the structural and functional homolog of the Cdc25 protein phosphatase (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Nagata *et al.*, 1991) and proof that at least two of these gene products are required for mitosis in HeLa cells (Millar *et al.*, 1991a; Galaktionov and Beach, 1991), provide confirmation that the key elements of the Cdc25/Wee1 mitotic control network first identified in fission yeast (Nurse, 1975; Fantes, 1979; Russell and Nurse, 1986, 1987) operate to regulate the initiation of mitosis in human cells.

The second important conclusion arising from these studies is that the human Wee1 protein directly carries out the inhibitory Tyr15 phosphorylation of the Cdc2 subunit of human p34^{cdc2}/cyclin B complex. Indirect evidence in favor of this conclusion was previously reported by Lundgren *et al.* (1991) who found that a functional *wee1* gene was required for Tyr15 phosphorylation of p34^{cdc2} in *mik1*$^{-}$ mutants of fission yeast. This was consistent with the data of Featherstone and Russell (1991), who proved that p107^{wee1} has an intrinsic tyrosyl kinase activity, and with the data of Parker *et al.* (1991) who found that fission yeast Wee1 promoted the *in vivo* tyrosyl phosphorylation of human p34^{cdc2} complexed to sea-urchin cyclin B in Sf9 insect cells. More recently it was reported that fission yeast p107^{wee1} promoted *in vitro* tyrosyl phosphorylation of metazoan forms of p34^{cdc2}/cyclin produced in Sf9 cells (Parker *et al.*, 1992). A curious feature of these reactions was that they were only reported to occur when the lysates of p107^{wee1} and p34^{cdc2}/cyclin expressing Sf9 cells were pre-mixed before co-precipitation, using either p13^{suc1}−Sepharose which precipitates a complex mixture of proteins, or glutathione−Sepharose in the case where GST−wee1 and p34^{cdc2}/GST−cyclin B cell lysates were used. Because this study did not report any experiments in which an attempt was made to phosphorylate purified p34^{cdc2}/cyclin with independently purified p107^{wee1}, it could not rule out the possibility that either another protein component in the cell lysates in addition to p107^{wee1} was required for tyrosyl phosphorylation of p34^{cdc2}/cyclin, or that the *in vitro* interaction between p107^{wee1} and p34^{cdc2}/cyclin from different species was so inefficient that it was dependent on the co-precipitation of both components on Sepharose at high concentrations. We have reported here that a highly purified form of human Wee1 phosphorylated two forms of human p34^{cdc2}/cyclin B on Tyr15 without the need for pre-incubation of mixed cell lysates or co-immobilization and concentration of the reaction components. The possibility of a contaminating or co-precipitating kinase being responsible for this phosphorylation reaction was eliminated by two experiments. In the first we demonstrated that the GST−Wee1 preparation phosphorylated sites equivalent to Tyr15 in synthetic peptides, and in the second we used the Arg114 mutation of Wee1 to prove that this phosphorylation was specifically dependent on the Wee1 kinase activity. These data, coupled with the demonstrated *in vivo* relationship between Wee1 activity and p34^{cdc2} tyrosyl phosphorylation (Lundgren *et al.*, 1991; Parker *et al.*, 1991) provide rigorous evidence that Wee1 kinases, although of the Ser/Thr-specific sequence homology class, actually carry out as their primary *in vivo* function the tyrosyl phosphorylation of p34^{cdc2}.

The third major aim of these studies was to determine whether human Wee1 kinase carried out the inhibitory Thr14 phosphorylation of p34^{cdc2}. We found that GST−Wee1 had no detectable ability to phosphorylate Thr14, either in p34^{cdc2}/cyclin B or in synthetic peptides. Likewise, fission yeast p107^{wee1} phosphorylated Tyr15 of synthetic peptides but had no activity against Thr14. We cannot eliminate the possibility that GST−Wee1 produced and purified from Schneider cells lacks some modification or additional component necessary for Thr14 phosphorylation, but this seems improbable. The most reasonable conclusion is that another kinase other than Wee1 carries out the Thr14 phosphorylation. This leads to the conclusion that in human cells there are two separate kinase pathways phosphorylating distinct inhibitory sites on p34^{cdc2}. The regulatory strategy

behind this mechanism of control is presently unknown. It is possible that the control processes regulating the activation of p34^{cdc2}/cyclin B operate not only by modulating Cdc25 as has been suggested by Enoch and Nurse (1991) and Kumagai and Dunphy (1992), but also by inhibiting Wee1 and the Thr14 kinase. If so, this provides two separate kinase pathways for the transmission of the large array of signals that impinge on the mitotic control, such as cellular growth, growth factors and cell cycle checkpoints.

Materials and methods

HeLa cell culture and transfection

HeLA cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. The plasmid for transfection was constructed in the mammalian expression vector pCMUIV using the *Bam*HI sites that were introduced to make GST−Wee1 (see below). This allowed insertion of the *WEE1* cDNA in both the correct orientation and reverse orientation with respect to the α-globin gene promoter. The pCMUIV vector containing the *E.coli* β-galactosidase gene (Hall *et al.*, 1983) was a gift of E.Joly (ICRF, Clare Hall, Cambridge, UK). Transfections were carried out as described by Paabo *et al.* (1986). 25 μg of DNA was used to transfect cells in a 100 mm tissue culture plate. At the indicated times cells were fixed in 2% formaldehyde for 10 min on ice and then stained for β-galactosidase activity by incubation overnight at 37°C in 5 mM ferrous cyanide, 5 mM ferric cyanide, 2 mM magnesium chloride, 0.2% NP-40, 0.1% sodium deoxycholate in phosphate buffered saline containing 1 mg/ml X-Gal.

Production and purification of GST−Wee1 and GST−Wee1-KR

A human *WEE1* cDNA suitable for cloning into pGEX-2T (Pharmacia, Inc.) was generated using the original clone pWEE1Hu (Igarashi *et al.*, 1991) as a template for PCR. The oligonucleotide primers 5′-CGCGGATCCATGGATACAGAAAAATCAGG-3′ and 5′-CGCGGATCCTCAGTATATAGTAAGGCT-3′ were used to generate a PCR fragment having *Bam*HI sites shortly upstream and downstream of the open reading frame. The PCR fragment was digested with *Bam*HI and ligated into pGEX-2T cut with *Bam*HI. A DNA fragment encoding Wee1 flanked by *Xho*I sites was produced by PCR with the primers 5′-GAGCTCGAGCCACCATGTCCCCTATACTAGG-3′ and 5′-CGCCTCGAGTCAGTATATAGTAAGGCT-3′. This PCR DNA product was cloned into the *Sal*I site downstream of the metallothionein promoter in the *Drosophila* expression vector pRMHa3 (Bunch *et al.*, 1988).

At a latter stage in these studies site-directed mutagenesis was used to generate pCMU−WEE1-KR and GST−Wee1-KR. This was done in two steps of PCR using *WEE1* as a template, the oligonucleotides described above and the oligonucleotide 5′-TTTCGATCGCCTAATGGCATAAAT-3′ which changes codon 114 from Lys to Arg and introduces a novel *Pvu*I site which was used to select mutant clones. All constructs were sequenced in their entirety to ensure that no extraneous mutations were introduced in the PCRs.

Schneider *Drosophila* cells were cultured at 27°C in Schneider medium (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics. Twenty-four micrograms of pRMHa3(GST−Wee1) was mixed with 1 μg of the selection plasmid phshsneo (Steller and Pirrotta, 1985) and used to transfect 1 × 10^7 Schneider cells by the calcium phosphate method. After 48 h cells were transferred to medium containing 0.5 mg/ml Geneticin (G418) (Gibco/BRL). Four weeks later stable populations of G418 resistant cells were obtained. To induce expression of protein, $CuSO_4$ (1 mM final concentration) was added to the medium and cells were grown for a further 48 h. Negative control transfectants were generated using pRMHa3 having an irrelevant gene, human T cell receptor T8, instead of GST−Wee1. Plasmids pRMHa3, phshsneo, PRMHa3-T8, and Schneider cells were provided by M.R.Jackson (TSRI). Cells were harvested by centrifugation and either frozen quickly or processed directly. Approximately 100 μl of packed cells were extracted in 1 ml lysis buffer (1% NP-40, 50 mM Tris pH 8.0, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.1 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, pepstatin and aprotinin) for 15 min at 4°C. A supernatant fraction was prepared by centrifugation at 15 000 *g* for 10 min at 4°C. Extract was mixed with GSH−Sepharose (Pharmacia) for 1 h at 4°C, washed with lysis buffer containing 0.5 M NaCl and washed with kinase assay buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 1 mM DTT). GST−Wee1 was eluted in kinase assay buffer containing 10 mM glutathione.

Kinase assays, phosphopeptide mapping and phospho-amino acid analysis

Kinase assays were carried out in 40 μl containing 10 μCi[γ-^{32}P]ATP for 15 min at 30°C. Reactions containing protein substrates were terminated by addition of an equal volume of SDS sample buffer, boiled and analyzed by SDS−PAGE. Reactions containing peptide substrates were terminated by addition of 30% acetic acid and rapid freezing, products were analyzed by thin layer electrophoresis. Angiotensin II (Calbiochem) was present at 0.625 mg/ml (0.9 mM) final concentration, TY peptide (a gift from K.Gould and P.Nurse, ICRF), as described by Gould and Nurse (1989) was present at 0.25 mg/ml (0.125 mM), 6−20 and mutant peptides (a gift from H.-C.Cheng and J.H.Wang, University of Calgary) were present at 0.125 mM. Purified human p34^{cdc2}/cyclin B from Sf9 cells was a gift of J.Y.Wang (UCSD) (R.Baskaran, S. Grunwald, and J.Y.Wang, manuscript in preparation). Purified p60^{src} kinase was a gift of T.Hunter (Salk Institute). T cell phosphatase was a gift of E.Fischer (University of Washington). Protein phosphatase $2A_c$ was purified from rabbit skeletal muscle (Cohen *et al.*, 1988). Phospho-amino acid analysis and peptide mapping were performed as described by Boyle *et al.* (1991).

Antibodies

Polyclonal rabbit cyclin B antibody was raised to bacterially produced HeLa cyclin B protein essentially as described by Pines and Hunter (1989) and was purified using the bacterially produced cyclin-B immobilized on CNBr−Sepharose (Pharmacia). Anti-C-terminal cdc2 rabbit polyclonal antibody was raised to a synthetic peptide corresponding to C-terminal residues (DNQIKKM) of human CDC2 (Lee and Nurse, 1987). Polyclonal rabbit *S.pombe* cdc2 was raised to bacterially produced p34^{cdc2} protein essentially as described by Gould and Nurse (1989). FITC-conjugated goat anti-rabbit IgG was from Cappel Inc.

Acknowledgements

We are very grateful for the assistance of the following people: H.Okayama for providing the human WEE1 clone prior to publication, M.Jackson for advice, assistance and materials in the use of Schneider cells, T.Hunter and J.Pines for advice and assistance with peptide mapping and supplying p60^{src}, R.Baskaran and J.Y.Wang for providing purified p34^{cdc2}/cyclin B, E.Joly for pCMU-β-galactosidase, K.Gould and P.Nurse, H.-C.Cheng and J.H.Wang for peptides. A-Chapman for technical help, G.Lenaers for the cdc2−6his construct, J.Millar for antiserum to *S.pombe* cdc2 and valuable advice, and Carol Featherstone for advice and encouragement. This work was supported by NIH grant GM-41281 awarded to P.R.

References

Boyle,W.J., Van Der Geer,P. and Hunter,T. (1991) *Methods Enzymol*, **210**, 110−149.
Bueno,A. and Russell,P. (1992) *EMBO J.*, **11**, 2167−2176.
Bunch,T.A., Grinblat,Y. and Goldstein,L.S.B. (1988) *Nucleic Acids Res.*, **16**, 1043−1060.
Cohen,P., Alemany,S., Hemming,B.A., Resink,T.J., Stralfors,P. and Lim Tung,H.Y. (1988) *Methods Enzymol.*, **159**, 390−408.
Draetta,G., Luca,F., Westerndorf,J., Brizuela,L., Ruderman,J. and Beach,D. (1989) *Cell*, **56**, 829−838.
Dunphy,W.G. and Kumagai,A. (1991) *Cell*, **67**, 189−196.
Dunphy,W.G., Brizuela,L., Beach,D. and Newport,J. (1988) *Cell*, **54**, 423−431.
Enoch,T. and Nurse,P. (1991) *Cell*, **65**, 921−923.
Fantes,P. (1979) *Nature*, **279**, 428−430.
Featherstone,C. and Russell,P. (1991) *Nature*, **349**, 808−811.
Galaktionov,K. and Beach,D. (1991) *Cell*, **67**, 1181−1194.
Gautier,J., Norbury,C., Lohka,M., Nurse,P. and Maller,J. (1988) *Cell*, **54**, 433−439.
Gautier,J., Minshull,J., Lohka,M., Glotzer,M., Hunt,T. and Maller,J.L. (1990) *Cell*, **60**, 487−494.
Gautier,J., Solomon,M.J., Booher,R.N., Bazan,J.F. and Kirschner,M.W. (1991) *Cell*, **67**, 197−211.
Gould,K.L. and Nurse,P. (1989) *Nature*, **342**, 39−45.
Gould,K.L., Moreno,S., Tonks,N.K. and Nurse,P. (1990) *Science*, **250**, 1573−1576.
Hall,C.V., Jacob,P.E., Ringold,G.M. and Lee,F.J. (1983) *Mol. Appl. Genet.*, **2**, 101−109.
Hartwell,L. and Weinert,T. (1989) *Science*, **246**, 629−634.
Hunter,T. and Cooper,J.A. (1985) *Annu. Rev. Biochem.*, **54**, 897−930.

Igarashi,M., Nagata,A., Jinno,S., Suto,K. and Okayama,H. (1991) *Nature*, **353**, 80–83.
Krek,W. and Nigg,E.A. (1991a) *EMBO J.*, **10**, 305–316.
Krek,W. and Nigg,E.A. (1991b) *EMBO J.*, **10**, 3331–3341.
Kumagai,A. and Dunphy,W.G. (1991) *Cell*, **64**, 903–914.
Kumagai,A. and Dunphy,W.G. (1992) *Cell*, **70**, 139–151.
Lee,M.G. and Nurse,P. (1987) *Nature*, **327**, 31–35.
Lee,M.S., Ogg,S., Xu,M., Parker,L.L., Donoghue,D.J., Maller,J.L. and Piwnica-Worms,H. (1992) *Mol. Biol. Cell.*, **3**, 73–84.
Lindberg,R.A., Quinn,A.M. and Hunter,T. (1992) *Trends Biochem. Sci.*, **17**, in press.
Lundgren,K., Walworth,N., Booher,R., Dembski,M., Kirschner,M. and Beach,D. (1991) *Cell*, **64**, 1111–1122.
Millar,J.B.A. and Russell,P. (1992) *Cell*, **68**, 407–410.
Millar,J.B.A., Blevitt,J., Gerace,L., Sadhu,K., Featherstone,C. and Russell,P. (1991a) *Proc. Natl. Acad. Sci. USA*, **88**, 10500–10504.
Millar,J.B.A., McGowan,C.H., Lenaers,G., Jones,R. and Russell,P. (1991b) *EMBO J.*, **10**, 4301–4309.
Nagata,A., Igarashi,M., Jinno,S., Suto,K. and Okayama,H. (1991) *New Biol.*, **3**, 959–968.
Norbury,C., Blow,J. and Nurse,P. (1991) *EMBO J.*, **10**, 3321–3329.
Nurse,P. (1975) *Nature*, **256**, 547–551.
Nurse,P. (1990) *Nature*, **344**, 503–508.
Paabo,S., Weber,F., Nilsson,T., Schaffner,W. and Peterson,P.A. (1986) *EMBO J.*, **5**, 1921–1927.
Parker,L.L., Atherton-Fessler,S., Lee,M.S., Ogg,S., Falk,J.L., Swenson,K.I. and Piwnica-Worms,H. (1991) *EMBO J.*, **10**, 1255–1263.
Parker,L.L., Atherton-Fessler,S. and Piwnica-Worms,H. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2917–2921.
Pines,J. and Hunter,T. (1989) *Cell*, **58**, 833–846.
Pines,J. and Hunter,T. (1991) *J. Cell Biol.*, **115**, 1–17.
Russell,P. and Nurse,P. (1986) *Cell*, **45**, 145–153.
Russell,P. and Nurse,P. (1987) *Cell*, **49**, 559–567.
Russell,P., Moreno,S. and Reed,S.I. (1989) *Cell*, **57**, 295–303.
Sadhu,K., Reed,S.I., Richardson,H. and Russell,P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5139–5143.
Steller,H. and Pirrotta,V. (1985) *EMBO J.*, **4**, 167–171.
Strausfeld,U., Labbé,J.C., Fesquet,D., Cavadore,J.C., Picard,A., Sadhu,K., Russell,P. and Dorée,M. (1991) *Nature*, **351**, 242–245.
Zoller,M.J., Nelson,N.C. and Taylor,S.S. (1981) *J. Biol. Chem.*, **256**, 10837–10842.

Received on July 20, 1992